A NEW SEQUENCE-SPECIFIC ENDONUCLEASE FROM A THERMOPHILIC CYANOBACTERIUM, *MASTIGOCLADUS LAMINOSUS*

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1. Introduction

The screening of a number of cyanobacteria (blue-green algae) for new sequence-specific deoxyribonucleases has been very rewarding [1–8]. We report here the purification of a new enzyme of this type from *Mastigocladus laminosus* (named *Mla* I) which recognizes and cleaves the nucleotide sequence TT↓CGAA.

2. Materials and methods

The strain used (CCAP 1447/1) was obtained from the Culture Centre of Algae and Protozoa (Downing St, Cambridge) and originated from hot springs in New Zealand. It was grown in aqueous medium D [9] containing (mg/l): nitrilotriacetate, 100; Na_2HPO_4, 110; $NaNO_3$, 700; KNO_3, 100; NaCl, 8; $MgSO_4 \cdot 7H_2O$, 100; $CaCl_2$, 50; $FeCl_3$ complexed with EDTA, 0.3 and trace amounts of Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, MoO_4^{2-} and BO_3^{3-} [5] buffered with 10 mM 2-[4-(2-hydroxyethyl)-piperazinyl(1)] ethane sulfonate (Hepes) at pH 8.1.

A 1500 ml cylindrical culture vessel containing this medium was placed in a waterbath at 45°C and sterile air was blown in from below. Illumination was done by 2 fluorescent lamps at 40 cm distance (~1500 Lux). The inoculum was grown in 3 large (14 cm diam.) Petri dishes with medium solidified with 1.5% agar at 37°C. It took 3 weeks to prepare 10 g cells/l liquid culture from this inoculum.

The various materials and methods used for determining the properties of the cleavage site of the *Mastigocladus laminosus Mla* I enzyme were as in [5,6].

2.1. *Assay of* Mla *endonuclease activity*

Mla I enzyme (column fraction, 8–15 μl) and 1 μg bacteriophage λ DNA were added to incubation mixtures (30 μl) containing 6.7 mM Tris–HCl (pH 7.4), 6.7 mM $MgCl_2$ and 6.7 mM mercaptoethanol. In the case of purified enzyme, KCl was present at 60 mM. Incubations were for 60 min at 40°C before gel electrophoretic analysis as in [5]. The presence of exonuclease activity was monitored by inspecting the width and sharpness of the bands after extensive incubation (8 h) of λ DNA with *Mla* I enzyme.

2.2. *Enzyme purification*

The initial stages of the purification procedure were as in [5,6] except that the material from the French pressure cell was diluted with 6 vol. buffer A (10 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol) in a stainless steel beaker immersed in an ice-bath and sonicated for 5 min with intermittent cooling. The deep blue solution (crude enzyme fraction) was brought to 0.1 M NaCl; polyethylene imine (Polymin P) at pH 7.9 was stirred in to 1% final conc. The insoluble residue obtained after centrifugation [5] was extracted 3 times with 0.6 M NaCl in buffer A (25 ml/100 ml crude enzyme fraction); the pooled extracts were dialyzed overnight against 2 × 2 l buffer B (20 mM KPO_4, (pH 7.4), 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol) at 4°C. The supernatant solution resulting from the Polymin P step was saturated to 70% with ammonium sulphate; the precipitate collected by centrifugation at 20 000 × *g* for at least 60 min was dissolved in 25 ml buffer B/500 ml crude enzyme fraction. This fraction was similarly dialyzed and combined with the dialyzed enzyme from the

0.6 M NaCl extraction step. The enzyme was percolated through a 20 × 2.5 cm column of phosphocellulose (Whatman P11) equilibrated with buffer B. The pass-through fraction containing the *Mla* endonuclease was then applied to a 15 × 2 cm column of DEAE cellulose (Whatman DE-52) equilibrated with buffer A. A 200 ml gradient of 0–0.30 KCl in buffer Ag (buffer A with 10% glycerol) was applied; the *Mla* I endonuclease appeared halfway through the gradient. The fractions of highest activity and quality were rechromatographed on a 10 × 1 cm column of DEAE-cellulose; the most active fractions were subsequently concentrated on a 5 × 0.5 cm column of DEAE-cellulose and eluted with 0.25 M KCl in buffer Ag. The enzyme solution at this stage still contains a blue pigment.

2.3. *Determination of enzyme cleavage specificity*

This was done in 2 complementary ways, both using the susceptibility of the *Hin*dIII/F fragment (~3500 basepairs long) of adenovirus 7 DNA [10] to the action of endo R· *Mla* I. The first approach is schematically outlined in fig.1. The above fragment was cleaved with endo R· *Hin*fI. The *Hin*fI-6 fragment (~200 nucleotides long) containing a cleavage site for *Mla* I at some 70 nucleotides from 1 terminus was labeled at both 5′-ends with ^{32}P using T4 polynucleotide kinase. One of the labeled termini was removed by incubation with endo R· *Hha* I and subsequent gel electrophoresis. A 5% aliquot of the fragment containing the endo R· *Mla* I site was incubated with this enzyme. It was subjected to slab gel electrophoresis in lane 5 next to a sequence ladder prepared from the remaining 95% with the chemical modification procedure in [11] of that fragment. This method allows a direct localization and characterization of the cleavage site for *Mla* I [12].

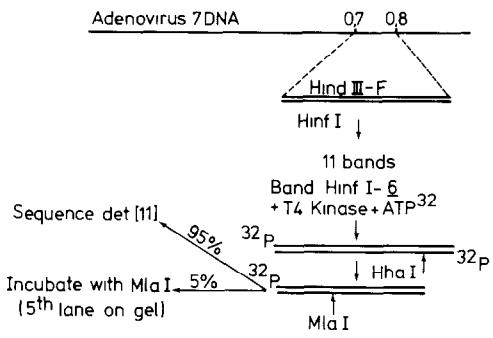

Fig.1. Design of experiment to establish cleavage site for endo R· *Mla* I.

The second approach was done in reverse in that the *Hin*dIII/F fragment was first labeled at the *Mla* site(s) and then cut with endo R· *Hin*fI to yield the 2 parts of *Hin*fI-6, each labeled with ^{32}P only at the terminus created by *Mla* I. These two fragments were each partially cleaved with pancreatic DNase to give a set of ^{32}P-labeled di-, tri- and higher oligonucleotides. Their structures were inspected for common sequences at the labeled termini and also compared with the results of the first approach. Similar experiments were done with 2 fragments of bacteriophage λ DNA.

3. Results

The purified *Mla* I enzyme was active over 30–55°C. Above the latter temperature our enzyme preparation caused degradation of DNA, but did not give discrete fragments.

The number of cuts made into various DNA preparations was comparatively small. None of the following small circular DNA molecules were cleaved: plasmid pBR322, bacteriophage φX174 RF DNA and Simian virus 40 (SV40) DNA. The DNA of bacteriophage λ was cleaved at 7 sites, while adenovirus 7 DNA is cut at 3 locations, one being present in *Hin*dIII/C (coordinates 0.5–0.64) and 2 in *Hin*dIII/F (coordinates 0.7–0.8) [10].

The results regarding the recognition and cleavage site of endo R· *Mla* I were the following. The outcome of the first approach (see section 2.3) show (fig.2) that the *Mla* I site resides in a sequence of the structure:

5′..GGGGAGTTCGAAGTAGAT..3′ (actual read off)

3′..CCCCTCAAGCTTCATCTA..5′

in which the cleavage site is situated close to the G or C (probably left of C) in a symmetrical hexanucleotide TTCGAA. Determination of the exact position of this site for the upper strand leads logically to that in the lower strand because of the palindromic nature of the double stranded hexanucleotide.

The results obtained with the second approach are documented in fig.3 which gives a 'wandering spot' analysis of the same nucleotide sequences at the 5′-termini created by endo R· *Mla* I. Fig.3A shows the sequence for the upper strand given above (5′ CGAAGTAGAT 3′), while fig.3B gives that for

Fig.2. Nucleotide sequence in Ad7 *Hin*dIII-F/*Hin*fI-6 fragment at both sides of point of cleavage by *Mla* I. Note that the structure of the enzymatic product (*Mla* I lane) is of the nature 5′ *p.....G G G G A G T T -OH′ 3′ which migrates slightly slower than the chemically degraded product of the same sequence 5′ p....G G G G A G T T -p 3′ carrying a 3′-terminal phosphate [12]. For experimental details see section 3. The T-lane is rather weak.

the lower strand (5′ CGAACTCCCC 3′). When the same approach was applied to 2 fragments of phage λ DNA labeled at the *Mla* I site the following [^{32}P]oligonucleotides were seen: pCpG, pCpGpA, pCpGpApA (in either of these fragments) as well as pCpGpApApT in one fragment and pCpGpApApG in the other. It follows that the cleavage site in the sequence TT↓CGAA is as indicated by the arrow.

4. Discussion

The results obtained from the two approaches all lead to the conclusion that the recognition and cleavage sequence of *Mla* I is represented by:

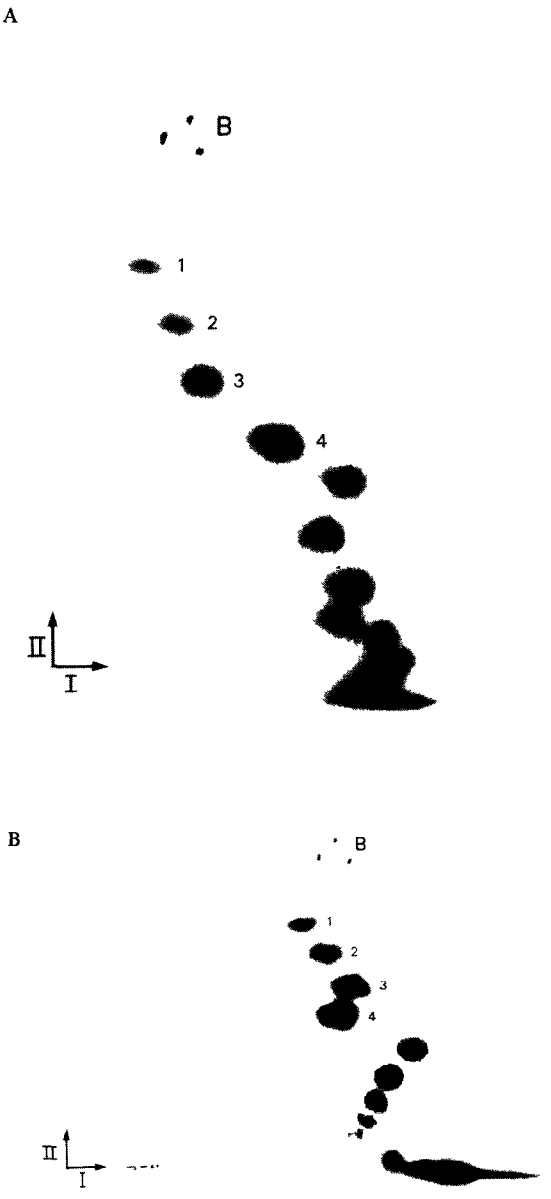

Fig.3. Nucleotide sequences of 5′-termini introduced into fragment of fig.2 by *Mla* I. Spot (1) was identified by venom phosphodiesterase treatment and electrophoretic comparison of the 5′-end labeled mononucleotide (pC) with markers. The other spots were identified by their relative mobilities. (A) Analysis of upper strand depicted in section 3 by the wandering spot method. (1)pCpG; (2)pCpGpA; (3)pCpGpApA; (4)pCpGpApApG; further additions to the sequence are T, A, G, A, T. (B) Analysis of lower strand (1)pCpG; (2)pCpGpA; (3)pCpGpApA; (4)pCpGpApApC; further additions to the sequence are T, C, C, C, C. B = blue marker, I = direction of electrophoresis, II = direction of homochromatography.

5′ TT↓CGAA 3′

3′ AAGC↑TT 5′

Nucleotides outside this sequence apparently do not contribute to the specificity of recognition. It is also very unlikely that the structure of this hexanucleotide could be degenerate. For example replacement of one pyrimidine and one purine would create hexamer TCCGGA which occurs once in pBR322 DNA ([14] and personal communication from C. Fuchs). This sequence is ruled out as *Mla* I site because this plasmid is not susceptible to attack by *Mla* I. Similarly, CTCGAG can not be a structure recognized by *Mla* I since this sequence occurs once in ϕX174 RF DNA [13] which is not a substrate for this enzyme. The low frequency of cleavages into large genomes (λ DNA, Ad7 DNA) also hints at the uniqueness of the *Mla* I recognition site.

Recently we have identified a second sequence-specific endonuclease in *Anabaena subcylindrica* (*Asu* II) which differs from *Asu* I in [3] and which also recognizes and cleaves the hexanucleotide sequence TT↓CGAA; this will be documented elsewhere.

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